Preserved leukocyte CD11b expression at the site of interstitial inflammation in patients with high-flux hemodiafiltration

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The impact of high-flux hemodialysis on clinical outcomes remains controversial. We have previously shown that *in vivo* transmigrated leukocytes from patients with low-flux bioincompatible hemodialysis have an impaired capacity to upregulate CD11b at the site of interstitial inflammation. In the present study, we investigated the *in vivo* capacity of transmigrated monocytes and granulocytes to express CD11b at the site of interstitial inflammation in 10 patients on biocompatible high-flux hemodiafiltration or high-flux hemodialysis and 12 healthy subjects, and the *in vitro* response to a bacteria-related peptide (*N*-formyl-methionyl-leucyl-phenylalanine (fMLP)). Leukocyte formation of hydrogen peroxide (H_2O_2) and leukocyte apoptosis were also studied. In patients, both monocytes and granulocytes had a preserved capacity to express CD11b following *in vivo* transmigration to sites of interstitial inflammation, compared with cells from healthy subjects. Furthermore, monocytes and granulocytes from patients showed a preserved ability to respond to challenge with fMLP in the extravascular milieu. The intracellular killing capacity of leukocytes (H_2O_2 production) in the interstitium was similar as of cells from healthy subjects both before and after stimulation with fMLP. Following maximal receptor independent stimulation (phorbol 12-myristate 13-acetate), leukocytes from patients showed lower H_2O_2 production at the site of intense inflammation, compared with cells from healthy subjects. Finally, leukocyte apoptosis in interstitial inflammation was similar in patients and healthy subjects. We conclude that *in vivo* transmigrated leukocytes from patients on biocompatible high-flux hemodiafiltration or high-flux hemodialysis have a preserved capacity to express CD11b at the site of interstitial inflammation. This may have important biological implications.

Kidney International (2007) **71,** 582–588. doi:10.1038/sj.ki.5002090; published online 17 January 2007

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Received 27 June 2006; revised 28 October 2006; accepted 14 November 2006; published online 17 January 2007

KEYWORDS: adhesion molecules; hemodiafiltration; biocompatibility; high flux hemodialysis; apoptosis; respiratory burst

Infection is a common cause of morbidity and mortality in patients with end stage renal disease[1–3] and mortality due to sepsis occurs much more commonly among hemodialysis patients than in the general population.[4] The enhanced susceptibility to infection is multifactorial. Major contributing factors are metabolic and functional abnormalities of leukocytes caused by accumulation of uremic toxins that inhibit leukocyte function and bioincompatibility of the dialysis procedure.[5–9] Previous studies have shown that specific uremic retention solutes have inhibitory effects on immune-competent cells.[10,11] Apart from this, several reports have demonstrated altered leukocyte adherence to endothelial cells, impaired phagocytosis and chemotaxis, altered generation of reactive oxygen species and impaired intracellular killing of bacteria.[12]

The recruitment of circulating leukocytes into inflamed tissues depends on interaction between adhesion molecules on leukocytes and vascular endothelial cells. Tethering brings the leukocyte into transient contact with the vessel wall allowing it to sample the endothelium for the presence of factors that activate a secondary phase of integrin-mediated adhesion, before transmigration into the extracellular matrix of the tissue.[13] Chemokines contribute to leukocyte recruitment firstly by activating integrins and secondly by promoting the migration of adherent leukocytes across the endothelium and through the extracellular matrix.[14]

We have previously studied leukocyte activation and transmigration into induced sites of inflammation in the interstitium in patients with low-flux bioincompatible hemodialysis, by use of a skin suction chamber technique.[15,16] *In vivo* transmigrated monocytes and granulocytes from patients with low-flux hemodialysis showed an impaired capacity to upregulate the adhesion molecule CD11b in response to interstitial inflammation. CD11b is important for leukocyte function in terms of phagocytosis

and an impaired response might contribute to an increased susceptibility to infections observed in this group of patients.

A number of previous studies have suggested that type of dialysis membrane (low-flux or high-flux) is associated with differences in long-term outcome of patients undergoing hemodialysis, both in terms of morbidity and mortality.[17–20] One controlled clinical study demonstrated that synthetic high-flux dialyzers were associated with improvement in neutrophil function in the peripheral circulation.[21] We therefore aimed at investigating the *in vivo* capacity of transmigrating monocytes and granulocytes to upregulate CD11b in response to an induced interstitial inflammation in patients treated with biocompatible high-flux hemodiafiltration or high-flux hemodialysis. Furthermore, leukocyte intracellular killing capacity and leukocyte apoptosis were studied at the site of interstitial inflammation. We hypothesized that high-flux treatment preserves leukocyte performance at the actual site of function, namely at the extravascular site in the interstitium.

RESULTS

There were no significant differences in the number of monocytes and granulocytes in the peripheral circulation or at the sites of interstitial inflammation between patients and healthy subjects at any time point.

Expression of CD11b on monocytes

There were no significant differences in the expression of CD11b on monocytes in the peripheral circulation between patients and healthy subjects at times 0 and 10 h (Table 1). The baseline expression of CD11b at 4°C was three to five times higher on *in vivo* transmigrated monocytes in the intact skin blister at 0 h compared with in the peripheral circulation in both patients and healthy subjects ($P<0.01$ for both comparisons). There were no significant differences in the expression of CD11b on monocytes in patients and healthy subjects at the three sites of interstitial inflammation. In addition, the increase in CD11b expression following transmigration was similar in patients and healthy subjects.

In vitro activation of monocytes with *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) induced a significant increase in the CD11b expression on cells in the peripheral circulation and at the three sites of interstitial inflammation in both patients and healthy subjects (Table 1). The fMLP-induced increase in CD11b on monocytes in the peripheral circulation was similar in patients and healthy subjects both at time 0 h and at time 10 h. The CD11b expression on monocytes following fMLP stimulation was similar in cells from patients and healthy subjects both in the intact blister at time 0 h and at the sites of intermediate and intense inflammation (Table 1).

Expression of CD11b on granulocytes

The expression of CD11b on granulocytes in the peripheral circulation was similar in patients and healthy subjects at time 0 and 10 h (Table 2). *In vivo* transmigration of granulocytes to induced sites of interstitial inflammation induced a two to four times higher expression of CD11b compared with cells in the peripheral circulation ($P<0.01$), which was similar in patients and healthy subjects. There were no significant differences in the baseline granulocyte CD11b expression at 4°C between granulocytes from patients and healthy subjects at the three sites of interstitial inflammation (Table 2).

Stimulation of granulocytes with fMLP induced a significant increase in the expression of CD11b on cells in the peripheral circulation, both in patients and healthy subjects (Table 2). There were no significant differences in the upregulation of CD11b on granulocytes at the three sites of interstitial inflammation following challenge with fMLP between patients and healthy subjects.

Respiratory burst (H_2O_2 production)

Hydrogen peroxide (H_2O_2) production of monocytes in the peripheral circulation at time 0 h was similar in patients and healthy subjects (Table 1). The H_2O_2 production was slightly higher of monocytes in the peripheral circulation at 10 h in patients compared with cells from healthy controls ($P=0.051$). Following stimulation with fMLP or phorbol 12-myristate 13-acetate (PMA), there were no significant differences in the H_2O_2 production of monocytes in the peripheral circulation in patients and healthy subjects (Table 1).

At the site of intermediate inflammation, the H_2O_2 production of monocytes was similar in patients and healthy subjects both before and following stimulation with fMLP or PMA (Table 1). At the site of intense inflammation, the H_2O_2 production of monocytes was higher in cells from healthy subjects compared with cells from patients ($P<0.05$). Similarly, the H_2O_2 production following stimulation with fMLP or PMA was higher in monocytes from healthy subjects compared with in cells from patients at the site of intense inflammation ($P<0.05$, Table 1).

In the peripheral circulation at 0 and 10 h and at the site of intermediate inflammation in the interstitium, the H_2O_2 production of granulocytes from patients was similar as in cells from healthy subjects (Table 2). At the site of intense inflammation, the H_2O_2 production of granulocytes from patients was significantly lower than from granulocytes from healthy subjects. In addition, granulocytes from patients showed an impaired response to fMLP at the site of intense inflammation in terms of H_2O_2 production compared with cells from healthy subjects. Following the most potent receptor-independent stimulation with PMA, granulocytes from patients showed an impaired response in terms of H_2O_2 production both in the peripheral circulation and at the sites of interstitial inflammation compared with cells from healthy subjects (Table 2).

Table 1 | Monocyte CD11b expression at 4°C and following stimulation with fMLP (mean ± s.d.); respiratory burst unstimulated and following stimulation with fMLP or PMA (median and range) in the peripheral circulation and at the sites of interstitial inflammation

	Peripheral circulation		Interstitial inflammation		
	0 h	10 h	0 h	Intermediate	Intense
CD11b expression (4°C, MFI)					
Patients	8.6 ± 2.2	10.6 ± 5.4	31.2 ± 2.7	35.1 ± 8.7	29.7 ± 6.6
Healthy subjects	7.2 ± 2.2	8.2 ± 3.6	38.8 ± 6.2	41.9 ± 11.2	40.4 ± 12.5
P (ANOVA)	NS	NS	NS	NS	NS
CD11b expression following fMLP (MFI)					
Patients	46.6 ± 11.6	53.5 ± 16.9	62.4 ± 10.3	62.1 ± 17.2	58.8 ± 17.2
Healthy subjects	57.5 ± 18.7	65.5 ± 13.8	76.4 ± 17.3	84.2 ± 20.7	76.8 ± 18.3
P (ANOVA)	NS	NS	NS	NS	NS
Respiratory burst (MFI)					
Patients	5.5 (1.4–18.7)	7.9 (2.5–29.9)	NE	17.8 (2.6–61.2)	1.4 (0.6–4.5)
Healthy subjects	3.8 (2.3–5.7)	3.6 (2.4–7.6)	NE	3.5 (1.3–6.7)	4.1 (0.5–6.6)
P (Mann–Whitney *U*-test)	NS	0.051		0.088	< 0.05
Respiratory burst (fMLP, MFI)					
Patients	7.4 (2.3–20.4)	8.9 (1.7–35.8)	NE	23.7 (2.6–66.5)	1.8 (0.6–15.9)
Healthy subjects	6.6 (4.8–13.6)	6.2 (4.1–12.4)	NE	9.4 (3.6–28.6)	7.7 (0.8–29.8)
P (Mann–Whitney *U*-test)	NS	NS		NS	< 0.05
Respiratory burst (PMA, MFI)					
Patients	53.9 (14.1–70.4)	55.1 (21.3–76.7)	NE	19.8 (6.8–51.5)	9.6 (3.4–30.5)
Healthy subjects	48.9 (37.2–157.3)	53.1 (35.4–139.8)	NE	15.2 (6.1–43.7)	26.1 (1.8–57.7)
P (Mann–Whitney *U*-test)	NS	NS		NS	< 0.05

ANOVA, analysis of variance; fMLP, formyl-methionyl-leucyl-phenylalanine; MFI, mean fluorescence intensity; NE, not examined; NS, nonsignificant; PMA, phorbol 12-myristate 13-acetate.

Table 2 | Granulocyte CD11b expression at 4°C and following stimulation with fMLP (mean ± s.d.); respiratory burst unstimulated and following stimulation with fMLP or PMA (median and range) in the peripheral circulation and at the sites of interstitial inflammation

	Peripheral circulation		Interstitial inflammation		
	0 h	10 h	0 h	Intermediate	Intense
CD11b expression (4°C, MFI)					
Patients	7.7 ± 4.1	6.7 ± 2.2	37.2 ± 3.5	54.9 ± 13.8	47.1 ± 8.4
Healthy subjects	5.0 ± 2.2	5.3 ± 1.3	33.9 ± 9.3	68.9 ± 18.1	65.4 ± 13.3
P (ANOVA)	NS	NS	NS	NS	NS
CD11b expression following fMLP (MFI)					
Patients	82.3 ± 15.6	93.2 ± 20.6	120.0 ± 24.5	115.9 ± 27.8	107.5 ± 29.8
Healthy subjects	96.6 ± 42.1	96.3 ± 32.4	111.4 ± 39.3	144.1 ± 31.2	128.6 ± 26.4
P (ANOVA)	NS	NS	NS	NS	NS
Respiratory burst (MFI)					
Patients	6.0 (1.1–23.7)	6.6 (1.4–25.3)	NE	23.5 (2.7–84.1)	3.1 (0.15–9.7)
Healthy subjects	2.7 (1.6–3.6)	2.4 (1.6–4.4)	NE	6.7 (4.2–12.0)	7.9 (5.1–20.8)
P (Mann–Whitney *U*-test)	NS	NS		NS	< 0.05
Respiratory burst (fMLP, MFI)					
Patients	19.3 (3.1–46.8)	18.2 (3.1–72.0)	NE	40.2 (3.7–97.7)	5.7 (0.3–31.4)
Healthy subjects	18.6 (10.3–33.2)	15.2 (10.5–34.2)	NE	22.9 (15.8–25.5)	19.8 (14.3–70.4)
P (Mann–Whitney *U*-test)	NS	NS		NS	< 0.01
Respiratory burst (PMA, MFI)					
Patients	123.4 (27.9–187.8)	129.7 (41.6–161.6)	NE	85.4 (18.3–125.9)	41.0 (6.6–139.6)
Healthy subjects	212.0 (100.8–415.6)	188.2 (117.9–270.7)	NE	152.2 (121.0–220.6)	130.8 (94.6–224.3)
P (Mann–Whitney *U*-test)	< 0.01	< 0.01		< 0.001	< 0.001

ANOVA, analysis of variance; fMLP, formyl-methionyl-leucyl-phenylalanine; MFI, mean fluorescence intensity; NE, not examined; NS, nonsignificant; PMA, phorbol 12-myristate 13-acetate.

Leukocyte apoptosis

In both, the monocytes and granulocytes, we observed no significant differences in the percentage of apoptotic cells (Annexin V+ and Annexin V+ propidium iodide (PI)+) in the peripheral circulation at times 0 and 10 h between patients and healthy subjects (data not shown). Furthermore, there were no significant differences in the percentage of apoptotic cells in the blisters representing intermediate or intense inflammation between patients and healthy subjects (data not shown).

DISCUSSION

Patients on hemodialysis have an increased susceptibility to infectious diseases. Retrospective data indicate that patients on high-flux hemodialysis and those treated with biocompatible membranes have a lower risk of infections compared with patients treated with low-flux membranes and bioincompatible dialyzers.[1,17,18,22]

In this study, we show that monocytes and granulocytes that transmigrate *in vivo* through the endothelium to sites of induced inflammation in the interstitium in patients on high-flux hemodiafiltration or high-flux hemodialysis, have a preserved capacity to upregulate CD11b, an adhesion molecule that is important for leukocyte function and phagocytosis.[13,14] Furthermore, leukocytes that transmigrate to inflammatory sites in the interstitium of patients showed a preserved capacity to respond to a second signal, for example, stimulation with fMLP in terms of CD11b expression, compared with cells from healthy subjects. These findings are in contrast to our previous observations of impaired monocyte and granulocyte CD11b expression at sites of interstitial inflammation in patients on low-flux bioincompatible hemodialysis.[15,16]

The preserved capacity of both monocytes and granulocytes to express CD11b at the sites of interstitial inflammation in patients on high-flux hemodiafiltration or high-flux hemodialysis may have important biological consequences in terms of preserved performance of cytotoxic actions in which the CD11b molecule plays a key role.[23,24] The mechanisms behind this preserved leukocyte function in patients with high-flux biocompatible procedures can only be speculated upon. One possible explanation may be that there is a selective recruitment of monocytes and granulocytes with a high CD11b phenotype in patients on high-flux biocompatible hemodialysis. Another, more plausible, explanation is that circulating low-molecular plasma factors that accumulate in the serum of dialysis patients and that have the potential to inhibit essential functions of leukocytes are removed by high-flux convective hemodiafiltration or high-flux hemodialysis. Examples of such potentially inhibitory factors are immunoglobulin light chains, guanidines, p-cresol, and granulocyte inhibitory proteins.[6,7,8,10,11,12] The determination of such leukocyte inhibitory factors in plasma in patients on high-flux dialysis was beyond the scoop of this investigation. However, it has been suggested that the presence of low molecular weight inhibitors of leukocytes in the plasma of end-stage renal disease patients may explain, at least in part, why infections are the most common cause of hospitalization and the second most common cause of death in patients with kidney failure.[5,8,12]

Granulocytes and monocytes produce reactive oxygen species as part of the host defense against invading bacteria. These cells generate reactive species using a multicomponent enzyme complex, a process referred to as the respiratory burst. The hemodialysis procedure is associated with an increase in the production of reactive oxygen species, to a degree depending on the dialysis membrane material.[25] In the present investigation, there were no significant differences in the H_2O_2 production from leukocytes in the peripheral circulation or at the site of intermediate interstitial inflammation between patients and healthy subjects. However, at the site of intense inflammation, the H_2O_2 production from both monocytes and granulocytes was significantly lower in patients compared with cells from healthy subjects. Following stimulation of leukocytes with fMLP, H_2O_2 production from both monocytes and granulocytes collected from the site of intermediate inflammation was similar in patients and healthy subjects. However, following the most potent stimulation with PMA, the production of H_2O_2 from both monocytes and granulocytes collected from the site of intense inflammation was significantly lower in cells from patients compared with cells in healthy subjects. Together these results indicate that monocytes and granulocytes collected from the peripheral circulation and *in vivo* transmigrated cells at the sites of intermediate inflammation in patients on high-flux hemodiafiltration or high-flux hemodialysis have a preserved capacity to respond with an increase in H_2O_2 production. However, following the most potent stimulation with PMA, both *in vivo* transmigrated monocytes and granulocytes from the site of intense inflammation in patients showed a lower response compared with cells from healthy subjects. This indicates the presence of a dose–response phenomenon in terms of leukocyte function in this group of patients. Our findings are in accordance with results from Ward *et al.*[26] who found that oxygen radical production from neutrophils in the peripheral circulation normalizes during high-flux dialysis. It has been suggested that an unknown low-molecular-weight factor capable of priming respiratory burst in granulocytes may be removed by high-flux hemodialysis.[26,27]

Clearance of leukocytes via apoptosis from the site of infection is crucial for the coordinated resolution of inflammation. The balance between stimulating and attenuating apoptotic factors is necessary for the maintenance of an effective immune response without the harmful side effects of neutrophil activation. Previous studies have indicated that uremia is a state that induces apoptosis, but that this is normalized with continuous and high-flux blood purification modalities.[28,29] In the present study, neither in blood nor at the sites of intermediate and intense inflammation were there any significant differences in monocyte and granulocyte

apoptosis between cells collected from patients and healthy subjects. Thus, also in this respect, high-flux procedures seem to preserve leukocyte function. This is in accordance with previous studies showing that dialysis membrane characteristics independently affect leukocyte cell apoptosis.[30,31]

The impact of the more biocompatible dialysis membranes used in this study, compared with in our previous,[15,16] on leukocyte function may be important and remains to be elucidated. Several previous clinical and experimental studies have shown that leukocytes collected from patients on bioincompatible dialyzers are unphysiologically activated in the peripheral circulation and that this affects leukocyte function, adherence to endothelial cells and the capacity to migrate.[9,12,21,32,33] However, among the patients in the randomized controlled hemodialysis (HEMO) study, chronic high-flux biocompatible hemodialysis did not significantly affect outcomes, including the rates of infections.[3,19,25] By contrast, recent observational data suggest that hemodiafiltration may improve patient survival independent of a higher dialysis dose.[34]

In conclusion, this is the first study to show that monocytes and granulocytes that transmigrate *in vivo* from the peripheral circulation to the site of actual action, that is in the interstitium, in patients on high-flux biocompatible hemodiafiltration have a preserved capacity to upregulate CD11b compared with cells from healthy subjects. Furthermore, monocytes and granulocytes in the interstitium were able to respond to a second signal (fMLP) at the site of intermediate inflammation. This supports the view that high-flux biocompatible hemodiafiltration or high-flux hemodialysis may preserve leukocyte function better than low-flux bioincompatible membranes, which may have important biological advantages.

MATERIALS AND METHODS

Study population

Ten patients (seven males and three females) with a mean age of 64 years (range, 33–74 years), treated with high-flux hemodiafiltration (eight patients) or high-flux hemodialysis (two patients) were included in the study. Demographics, dialysis prescription, dialysis access, K_t/V, medication and infections are shown in Table 3. All patients had polysulfone membranes (F80-F100, Fresenius Medical Care, Germany). None of the patients were suffering from infectious diseases or active systemic inflammatory diseases and none were taking antibiotics, anti-inflammatory or immunosuppressive drugs. Mean hemodialysis time was 14 ± 1 h per week and mean K_t/V was 1.7 ± 0.3. The renal diagnoses were: inactive glomerulonephritis (four patients), nephrosclerosis (three), interstitial nephritis (two), and polycystic kidney disease in one patient. Twelve healthy subjects (six males and six females) with a mean age of 55 years (range, 32–75 years) were also studied. None of the healthy subjects were on any medication at the time of examination. Informed consent was obtained from all participants and the study was approved by the Ethics Committee of the Karolinska University Hospital, Stockholm, Sweden.

Skin suction chambers and collection of blister fluid

This method has previously been described in detail.[15] Briefly, a site of interstitial inflammation was induced on one of the forearms of patients and healthy subjects, by use of a skin suction chamber technique. A vacuum was applied by continuous suction and two blisters were raised within 2–3 h. The skin blisters were covered and 12–14 h thereafter the roofs were removed after the blister fluid had been aspirated, pooled, and saved for analysis in −70 C. The first pool of exudates collected from the two overnight blisters was designated '0 h'. Skin bond cement was then applied around the bottom edge of sterilized open-bottom plastic skin chambers with a volume of 1 ml. The skin chambers were placed over the unroofed blisters and secured. In the proximal chamber, 1 ml of heparinized autologous serum was added (6 μl Heparin added to 1 ml serum). The autologous serum was collected the day before, centrifuged for 15 min at 4°C and immediately frozen at −70°C. In the distal chamber, 1 ml phosphate-buffered saline (PBS) and 6 μl Heparin were inserted. The administration of serum and buffer in the respective chambers was carried out in order to induce two degrees of intensity of the inflammatory stimulus in the blisters. An 'intense inflammation' was induced in the chamber with serum and an 'intermediate inflammation' in the chamber containing buffer. The reason for the nomenclature is based on previously published data.[15,16] Patients were thereafter dialyzed for 4–5 h. After 10 h of incubation, the blister fluid in the respective skin chamber was aspirated and placed on ice. The blister fluid was then centrifuged at 1300 r.p.m. for 5 min at 4°C and the pellets were resuspended in 500–1000 μl of 0.15 M PBS of pH 7.4, supplemented with 0,9 mg/ml glucose (PBS-glucose) and kept on ice until used for analysis. The

Table 3 | Patient demographics, dialysis prescription, medication, and previous infections

							Medication				
Patient no.	Age	Time on dialysis (years)	Type	Hours/week	Access	K_t/V	EPO	Iv iron	Vitamin D	Statin	Infections during time on dialysis
1	33	2	HDF Pre 30 lit	15	AV-graft	1.7	+	+	+	+	Recurrent upper UTI
2	62	2	HD	15	AV-fistula	1.5	+	+	+	+	Access-related septicemia
3	69	3	HDF Post 20 lit	16	AV-fistula	1.3	–		+	+	Access-related septicemia
4	71	3	HDF Post 25 lit	13.5	AV-fistula	1.8	+	+	+	–	Access-related septicemia
5	68	3	HDF Post 24 lit	15	AV-fistula	1.7	+	+	+	–	Bacterial bronchitis Virosis
6	72	3	HDF Post 60 lit	13.5	AV-fistula	1.7	+	+	+	+	Pneumonia
7	64	5	HDF Post 20 lit	15	AV-fistula	1.7	+	–	+	–	Recurrent UTI Streptococci in leg ulcer
8	74	4	HDF Post 24 lit	13.5	AV-fistula	2.4	–	–	+	–	–
9	60	6	HD	12.5	AV-fistula	1.8	+	–	+	–	–
10	63	7	HDF Post 14 lit	13.5	CDC	1.4	+	+	+	–	–

CDC, central dialysis catheter; EPO, erythropoietin; HD, high-flux hemodialysis; HDF, hemodiafiltration (Pre, pre-dilution; Post, post-dilution, volume in litres); UTI, urinary tract infection.

reason for choosing 10 h of incubation was based on previous studies in which we and others have observed that a sufficient number of cells transmigrate during 10 h.[15,16,35]

Collection of blood samples

Blood samples were drawn in the morning from patients and healthy subjects, when the first pool of blister exudates was collected (see above) and 10 h thereafter. Samples were collected in tubes containing citrate (Vacutainer, 5 ml, with 0.129 M Na-citrate Becton Dickinson, Plymouth, UK). Blood samples were kept on ice to prevent further complement activation and modulation of adhesion molecule expression on monocytes and granulocytes.

Preparation of peripheral leukocytes

Erythrocytes were hemolyzed by addition of 2 ml 4°C isotonic NH_4Cl-ethylenediaminetetraacetic acid 'lysing solution' (containing 154 mM NH_4Cl, 10 mM $KHCO_3$, and 0.1. mM ethylenediaminetetraacetic acid, pH 7.2) to 100 μl blood. The samples were incubated at 4°C for 5 min and then centrifuged at 1300 r.p.m. for 5 min at 4°C to produce a leukocyte pellet. The leukocytes were finally washed in 2 ml 4°C PBS-glucose and centrifuged. Cells to be used for immunostaining were resuspended in 100 μl cold PBS with the addition of 0.02% sodium azide (PBS-azide) and cells to be analyzed for respiratory burst were resuspended with 100 μl cold PBS-glucose. The cells were kept on ice during handling and the experiments were performed within 30 min to avoid further cell activation.

Number of leukocytes in the peripheral circulation and at the sites of interstitial inflammation

In the blister exudates the number of monocytes and granulocytes were counted using a flow cytometer (Epics Elite, Beckman Coulter Inc., Hialeah, FL, USA). To determine the number of leukocytes in the peripheral circulation, 100 μl blood was hemolyzed, stabilized and fixed according to the Multi-Q-prep, ImmunoPrep technique (Beckman Coulter Inc.) and the cells were counted by flow cytometry (Beckman Coulter Inc.).

Analysis of leukocyte CD11b expression

Leukocytes from patients and healthy subjects were isolated from blood at 0 and 10 h and from the blister fluid at time 0 h, intermediate inflammation and intense inflammation at 10 h. In order to study the ability of leukocytes to respond to a second signal, monocytes and granulocytes were activated in the presence of 200 μl 0,5 μM fMLP (Sigma Chemical Co., Dorsett, UK) for 15 min at 37°C. Monocytes and granulocytes incubated with RPMI 1640 medium supplemented with 0.01 mmol/l *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES-RPMI-buffer, Gibco Ltd, Paisley, Renfrewshire, UK/Invitrogen AB) and 5% heat-inactivated fetal bovine serum for 15 min at 4°C, served as a negative control. Cells were then washed with 2 ml cold PBS-azide and centrifuged at 1300 r.p.m. at 4°C for 5 min. The supernatant was removed and the cells were resuspended in 100 μl PBS-azide. Five microliter of phycoerythrin-conjugated monoclonal mouse anti-human CD11b, C3bi receptor/RPE (Clone 2LPM19c, Dako AS, Glostrup, Denmark) was added to each sample before incubation for 30 min on ice. The cells were then washed with 2 ml cold PBS-azide, centrifuged at 1300 r.p.m. at 4°C for 5 min and the pellets were finally resuspended in 500 μl cold PBS-azide before analysis by flow cytometry. Using flow cytometry, the density of the adhesion molecule expression of granulocytes and monocytes was presented as mean fluorescence intensity of the gated leukocyte population within a chosen field.

Measurement of respiratory burst

Cells were isolated from blood at times 0 and 10 h and from the blister fluid representing intermediate inflammation or intense inflammation at 10 h and were incubated with 5 μM 2′,7′-dichlorofluorescein diacetate (Fluka 35847, Sigma Chemical Co., Dorsett, UK) in PBS-glucose for 15 min at 37°C. The samples were gently shaken at least three times during the incubation in order to get a proper permeabilization of 2′,7′-dichlorofluorescein diacetate and to avoid cell aggregation. Following incubation with 2′,7′-dichlorofluorescein diacetate, granulocytes and monocytes were stimulated with either 200 μl 0.5 μM PMA (D-5879, Sigma Chemical Co., Dorsett, UK) in PBS-glucose (receptor-independent stimulus) or 200 μl 0.5 μM fMLP (receptor-dependent stimulus) in PBS-glucose and incubated for 15 or 30 min, respectively, at 37°C. Cells incubated with 200 μl PBS-glucose alone for 15 min on ice were used as a negative control. The samples were gently shaken three times during the incubation. After incubation, the cell suspensions were placed on ice and 1 ml cold PBS-azide supplemented with 0.1 mM ethylenediaminetetraacetic acid was added to stop the reaction. The samples were analyzed immediately by flow cytometry and the amount of H_2O_2 formation, indirectly expressed as green fluorescence from the DCF molecules, was expressed as mean fluorescence intensity of the gated leukocyte population within a chosen field.

Analysis of leukocyte apoptosis

Monocytes and granulocytes from patients and healthy subjects were analyzed as to the percentage of apoptotic cells in the two different cell populations. Cells were isolated from blood at the two time points (0 and 10 h) and from the blisters representing intermediate and intense inflammation. We used the Annexin V kit from BD Biosciences (IM3546), which makes it possible to stain the cells both with Annexin V and PI. Simultaneous staining with Annexin V and PI serves to define cells that are in an intact viable state, an early apoptotic state and a late apoptotic or necrotic state, respectively. This is expressed as negative staining for both Annexin V and PI, positive staining for Annexin V but not for PI and positive staining for both Annexin V and PI, respectively. Cells were stained according to the manufacturer's instructions and immediately analyzed by flow cytometry. The percentage of early and late apoptotic cells was calculated for the two leukocyte populations.

Statistical analysis

Results are expressed as mean ± s.d. for the normally distributed data (CD11b) and as median and range for the non-parametric data. Statistical analysis was performed using ANOVA for repeated measurements and the post hoc Scheffé test for the CD11b analyses. Mann–Whitney *U*-test for two independent samples was used to analyze the data for the hydrogen peroxide formation and apoptosis, as results were not normally distributed and the variances were not homogenous.

ACKNOWLEDGMENTS

We thank Anette Bygdén and Titti Nieminen for skillful technical assistance. This study was supported by an unrestricted grant from the Karolinska Institutet, Stockholm Sweden and Terumo Europe N.V. *dedicated to well being*.

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